Exhibit 16



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labeled by random priming using 3 nmol of Cy3-dCTP or Cy5-dCTP (NEN, Boston, MA) and 0.6 nmol dCTP. For array hybridization, 0.5 μg of labeled tumor DNA was co-precipitated with 0.5 μg of normal control, 5 μg Cot-1 DNA, and 20 μg sheared salmon testis DNA. The probe mixture was dissolved in 5 μl $H_2O$, and SSPE and SDS were added to a final concentration of 6× SSPE and 2% SDS in a final volume of 15 μl. The probe solution was denatured at 98°C for 2 min, cooled to room temperature, and spread onto the array and covered with a 22 × 22 mm coverslip. The slide was enclosed in a Corning hybridization chamber and incubated at 62°C for 16–22 h. After hybridization, the array was rinsed with $H_2O$ twice at room temperature and then washed in 2× SSC, 0.5% SDS at 65°C with shaking for 45 min. The arrays were rinsed with $H_2O$ and air-dried right before scanning.

**Array image acquisition and data processing.** The fluorescent signals were scanned into image files using the Scan Array 3000 (General Scanning, Watertown, MA). Images were quantified using Imagen software (Biodiscovery, San Diego, CA). The relative fluorescent level or fluorescent ratio, representing the relative amounts of target sequences in the probe mix, was analyzed by comparing the fluorescent intensity of corresponding individual spots after background subtraction. When equal amounts of genomic probes labeled with Cy5 and Cy3 were comparatively hybridized, the variation of fluorescence ratio of the two colors was within 10% for different BACs on the array. The average local background and standard deviation over all the array spots was calculated. Local background value for a spot was replaced with the average background if it was 2 s.d. units above the average value. The mean ratio over all the unique clones was calculated, as well as the s.d. Those ratios beyond 1.5 s.d. from the mean were discarded from the calculation, and the mean and s.d. were recalculated. The final average ratio value was used as the normalization factor to normalize all the ratios. The ratios of clones were plotted along individual chromosomes for easy inspection. For each sample two experiments were done with reversal of the dye labels. The Cy5:Cy3 ratios were plotted together along individual chromosomes. The two ratio curves should be reciprocal; thus any ratio artifact can be easily identified. A BAC clone was judged to be significant only if two or all three replicate spots passed the criteria in both experiments.

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# Expression of exogenous protein in the egg white of transgenic chickens

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Using a replication-deficient retroviral vector based on the avian leukosis virus (ALV), we inserted into the chicken genome a transgene encoding a secreted protein, β-lactamase, under the control of the ubiquitous cytomegalovirus (CMV) promoter. Biologically active β-lactamase was secreted into the serum and egg white of four generations of transgenic chickens. The expression levels were similar in successive generations, and expression levels in the magnum of the oviduct were constant over at least 16 months in transgenic hens, indicating that the transgene was stable and not subject to silencing. These results support the potential of the hen as a bioreactor for the production of commercially valuable, biologically active proteins in egg white.

A large and rapidly increasing number of proteins are required in medicine and industry in kilogram or metric ton amounts per year. Many of these proteins assume a native conformation only in the correct cellular environment and require specific post-translational modifications. These protein products are typically produced in mammalian cells in industrial fermentation facilities. The low yield of recombinant protein from such cells, combined with high facility costs, have led to the search for alternative production systems.

A number of proteins, including biopharmaceuticals, have been produced in the milk of transgenic mice, rabbits, pigs, sheep, goats, and dairy cattle at concentrations of up to ten grams per liter[1–4]. Although promising, mammary gland bioreactors have several drawbacks, including long generation times. Goats, for example, take 18 months from the creation of founder transgenic embryos to the production of milk by progeny. The biochemical complexity of milk complicates the purification of recombinant proteins, and evolutionary conservation between humans and other mammals means that many pharmaceuticals may adversely affect the production animal.

A modern, genetically selected White Leghorn hen lays up to 330 eggs per year, each containing ~6.5 grams of protein. About 3.5

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Exhibit 16

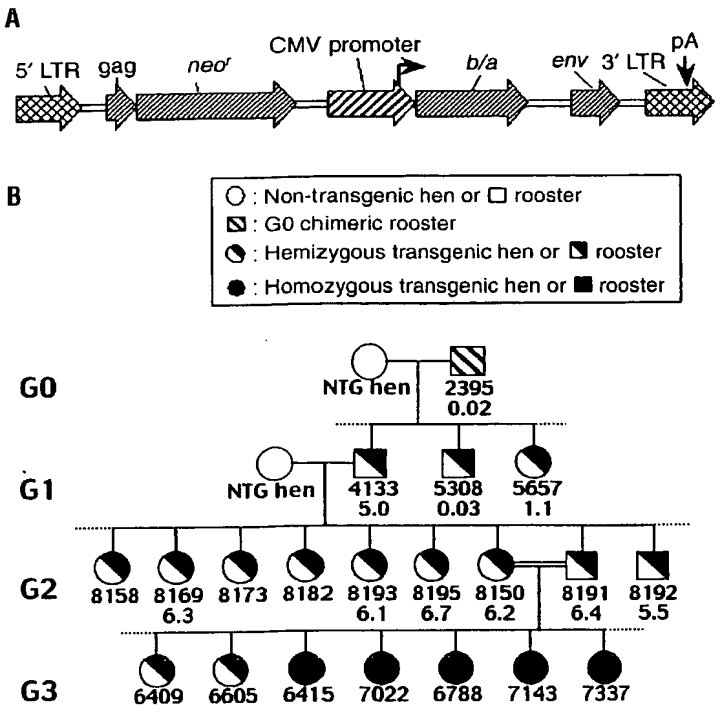


**Figure 1.** Vector organization and overview of transgenic lineages. (A) Schematic of NLB-CMV-BL. Sequences marked are 5′ and 3′ long terminal repeats (LTR), cytomegalovirus promoter (CMV), polyadenylation sequence (pA), genes for *neo′*, β-lactamase (*bla*) and remnants of *gag* and *env*. (B) Pedigree of chickens bearing the transgenic loci harbored by rooster 4133. Rooster 2395 carried multiple transgenic loci and was bred to a non-transgenic (NTG) hen, yielding 3 offspring, each carrying the transgene in a unique position of the chicken genome. For simplicity, transgenic progeny for which expression data were not presented and non-transgenic progeny were omitted from the pedigree. Band numbers are indicated below each symbol. Concentrations (μg/ml) of β-lactamase in the serum of some chickens are shown below band numbers.



grams of the total protein is egg white, of which ~90% is accounted for by seven genes; the *ovalbumin* gene alone accounts for 2 grams of egg white protein. Tubular gland cells line the lumen of the magnum of the oviduct and continually express and store egg white proteins in intracellular granules. As a yolk passes through the magnum, the granule contents are released and deposited onto the yolk. Advantages of protein production in chicken eggs include short generation times and prolific rates of reproduction via artificial insemination. In addition, the glycosylation patterns of IgGs in chickens in some aspects resemble those in humans more than do those of goats, cows, and sheep[5].

Use of the hen for protein production has been hampered by the lack of a robust method to genetically engineer the avian genome. Two promising efforts are underway but have met with limited success. The oocyte or the yolk can be isolated shortly after ovulation, and vector DNA can be injected in the vicinity of the pronuclei. Although carried episomally in most offspring, injected DNA has been observed to integrate, yielding a single mosaic rooster that, when outbred, generated G1 offspring hemizygous for the transgene[6]. The method is labor intensive, however, requiring that the hen donating the oocyte be killed. As an alternative, chimeras bearing transfected, pluripotent blastodermal cells can readily be produced[7], but this method has yet to yield a transgenic bird with germline transmission.

Retroviral vectors are able to introduce transgenes into the chicken genome at low but acceptable efficiencies. The first transgenic birds were produced using the reticuloendotheliosis virus (REV) and ALV[8]. These replication-competent vectors induced viremia in infected birds and thus could not be used for commercial applications. However, a replication-deficient REV vector was used to produce G1 transgenic birds expressing chicken growth hormone in the blood at levels from <1 to 254 ng/ml[9]. A replication-deficient ALV-based vector, termed NLB[10], was used to express a transgene through three generations. This system was limited by a low frequency of germline transmission, which was overcome by the development of a high-throughput screening procedure for transgenes[11]. Retroviral systems hold promise for oviduct bioreactors in that the vectors can carry tissue-specific promoters such as a 215-bp chicken $\alpha_{1k}$-actin promoter that conferred preferential expression in the skeletal muscle of infected chickens[12].

Here we show that a foreign, biologically active protein can be stably expressed in the egg white of transgenic hens. Because the elements necessary for tissue-specific expression in adult oviducts have not yet been determined, we chose to express β-lactamase from the ubiquitous CMV promoter. β-lactamase, which is expressed and secreted into the periplasm of TEM-1 type *E. coli*, was chosen as the reporter because it can be secreted from many types of eukaryotic cells and is easily assayed[13]. To create pNLB-CMV-BL, we replaced *lacZ* with the CMV promoter and β-lactamase coding sequence (Fig. 1A)[11].

To generate G0 transgenic birds, we injected 7–10 μl of concentrated virus into the subgerminal cavity of 546 White Leghorn stage X embryos, from which 126 chicks hatched. The efficiency of transgenesis was relatively low: the transgene was detected by PCR in the blood DNA of approximately 10% of hatched chicks. In the chicks positive for the transgene, 1% of blood cells harbored the transgene. The level of β-lactamase was measured in the serum and egg white by a kinetic colorimetric assay in which pyridine-2-azo-*p*-dimethylaniline cephalosporin (PADAC), a purple substrate, is hydrolyzed by β-lactamase. Of the G0 chickens, 9 had serum levels of β-lactamase ranging from 11.9 to 173.4 ng/ml at 6–7 months after hatching. Of 57 hens, 6 had significant levels of β-lactamase in egg white, ranging from 56.3 to 250.0 ng/ml.

Because G0 birds harbored the transgene in a fraction of their cells, those that harbored the transgene in their germ cells had to be identified and mated with non-transgenic partners. Blood DNA of offspring was screened for the transgene by a qualitative real-time PCR assay[11]; those that were positive carried one copy of the transgene in 100% of their somatic and germ tissues. Copy number was determined by Southern blot analysis and confirmed by Mendelian segregation of the transgene in progeny bred from G1 transgenic hens and roosters[11]. Accordingly, sperm DNA from 56 G0 roosters was screened for the presence of the transgene using a real-time PCR assay[11]. Three G0 roosters harbored low levels of the NLB transgene and were outbred. Rooster 2395 gave rise to 3 G1 transgenic offspring out of 422 progeny, whereas the other 2 G0 roosters yielded no transgenic offspring out of 630 total progeny. Serum from G1 transgenic chicks 5308, 5657, and 4133 at 4–9 weeks after hatching contained 0.03, 1.1, and 5.0 μg/ml of β-lactamase, respectively.

Rooster 4133 and hens 5308 and 5657 were bred to non-transgenic chickens to obtain hemizygous offspring. Pedigrees of the transgenic progeny of rooster 4133 and subsequent generations are

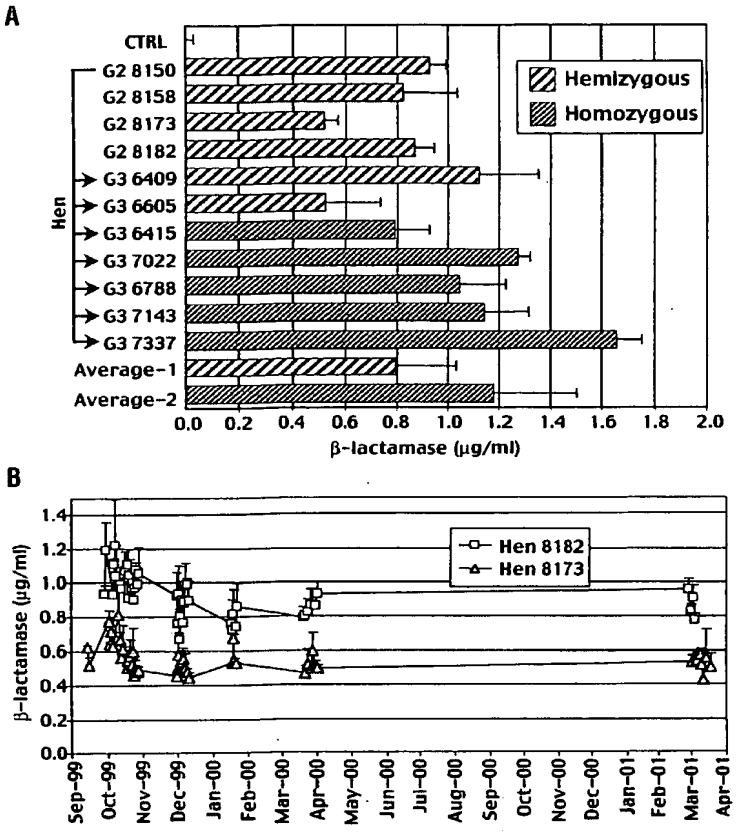


**Figure 2.** β-lactamase in the eggs of G2 and G3 hens bred from rooster 4133. (A) Egg whites from four representative G2 hemizygous transgenic hens and hemizygous and homozygous transgenic G3 hens bred from rooster 4133 were assayed for active β-lactamase. Eggs were collected when the hens were 25–41 weeks old. The control represents egg white from untreated hens and non-transgenic clutchmates. Arrows indicate G3 hens bred from G2 hen 8150. Band number and generation are indicated to the left. Transgene copy number is denoted by stripe pattern. Average concentration for hens carrying one or two copies is at the bottom of the chart. (B) β-lactamase expression in the eggs of transgenic hens over time. The first set of eggs laid by hens 8182 and 8173 and subsequent sets were collected periodically and assayed for active β-lactamase 1–2 months after collection. Each data point is the mean β-lactamase concentration for a single egg. For simplicity, negative error bars were removed.

shown (Fig. 1B). The progeny of 4133 had serum levels of β-lactamase between 5.0 and 6.7 μg/ml, whereas those of the progeny of 5657 were between 1.9 and 2.3 μg/ml; none of the offspring of 5308 had detectable β-lactamase in their serum or egg white. Expression of the transgene in serum was expected given the ubiquitous nature of the CMV promoter. Southern blot analysis indicated that the CMV-BL transgene was integrated intact in all three pedigrees (data not shown), suggesting that the transgene had been silenced during the course of germline transmission in this line. The 5308 line was not analyzed further.

It is critical for any bioreactor that transgene expression be stable within a single generation of production animals as well as between generations. To examine this, we crossed G1 hemizygous rooster 4133 and hen 5657 to nontransgenic partners and measured β-lactamase in the eggs of their female offspring. Results for hen 5657 and her progeny are summarized below. Fifteen transgenic hens bred from rooster 4133 had egg white β-lactamase at concentrations ranging from 0.47 to 1.34 μg/ml. Four representative hens are shown in Figure 2A. Expression levels were high in the initial eggs but leveled out after several months and remained essentially constant for up to 16 months (Fig. 2B).

G1 and G2 chickens carry a single copy of the transgene on one chromosome. To determine if doubling of the transgene copy number would increase expression of β-lactamase, G2 hen 8150 and sibling G2 rooster 8191 were crossed to yield hemizygous and homozygous G3 hens, and results were confirmed by quantitative PCR analysis[11]. The G3 hens expressed β-lactamase in egg white at concentrations ranging from 0.52 to 1.65 μg/ml. The average expression in eggs from homozygous hens was 47% higher than in eggs from hemizygous hens (Fig. 2A). This lower-than-expected increase was probably due to the variability between hens and the small number of hens analyzed. Western blot analysis confirmed that the transgene was faithfully producing intact β-lactamase in the eggs of G2 transgenics (Fig. 3). The β-lactamase level detected by western blot also correlated closely with that determined by the enzyme activity assay, indicating that most of the egg white β-lactamase was enzymatically active. In similar experiments with G1 hen 5657, egg white β-lactamase levels were the same in the hen and her progeny, although the average level was low: 0.13 to 0.2 μg/ml. Only one homozygous G3 transgenic hen was obtained for the 5657 lineage; its egg white β-lactamase levels were twice that of the levels from hemizygous G1 and G2 hens.

The hen has long been touted as a potential bioreactor for producing human pharmaceuticals in high yield and at low cost. Here we have provided evidence for the stable and continued production of a biologically active foreign protein in the egg white of transgenic hens. Expression levels remained nearly constant over many months and across successive generations, and the foreign protein was homogeneous with respect to molecular weight as determined by western analysis. The next step will be to develop more robust methods of transgenesis and expression vectors that will express high concentrations of a target protein solely in tubular gland cells of the oviduct.

To prevent viremia induced by complementation or recombination of the retroviral vector with endogenous retroviral loci, we used embryos from chickens free of active retroviruses in combination with a vector, NLB, from which 85% of the viral genome had been removed. No replication-competent viruses are produced in the cell lines used to package NLB[14–16]. As a result, we have not detected ALV in the serum of transgenic chickens, further validating the NLB system.

The hen has many advantages over other systems as a bioreactor. Unlike cows, goats, and sheep, chickens have been raised for many generations as specific pathogen–free animals in biosecure facilities. Unlike milk biologics, human and animal vaccines produced in eggs have a long regulatory history. The short generation times and prolific rates of reproduction allow production to be scaled up rapidly. In contrast to cell-based production systems, which operate most efficiently in batch mode, chickens continuously produce eggs year-round, allowing efficient use of downstream facilities for purification and processing. Finally, whereas mammary bioreactor companies target low-dose, high-potency drugs, such as blood clotting factors, the hen, because of its high-throughput and low-cost infrastructure, should be useful for producing high-dose, low-potency drugs, such as human serum albumin and antibodies.

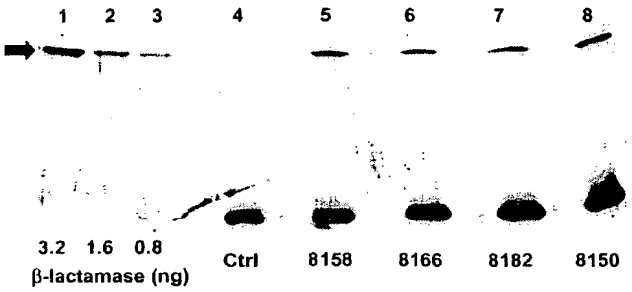


Figure 3. Western blot analysis of β-lactamase in the egg white of transgenic hens. β-lactamase in egg white samples from G2 hens was detected with an anti-β-lactamase antibody. Bacterially expressed, purified β-lactamase was electrophoresed in lanes 1–3 in amounts indicated below each lane. Lanes 4–8 contained 1.5 μL of egg white from a non-transgenic chicken (lane 4), and from transgenic G2 hens bred from rooster 4133 (lanes 5–8). The arrow indicates the position of β-lactamase. Hen numbers are listed below. The bands at the bottom of lanes 4–8 were due to an egg white protein that cross-reacted with the anti-β-lactamase antibody and is approximately 13 kDa. Hens were 23 weeks of age when the eggs were collected.

## Experimental protocol

Production and testing of transgenic chickens. Details of vector construction and of the production of transduction particles and transgenic chickens are described in ref. 11. To check for the presence of replication-competent ALV of subgroups A, B, and J, we drew serum from adult chickens and cultured it on DF-1 cells[17,18]. After 6 days of culture, media and cell lysates were tested with a p27 enzyme-linked immunosorbent assay (ELISA; FlockChek ALV Test Kit, IDEXX, Westbrook, ME). To date, no ALV has been detected in any transgenic chicken.

β-lactamase assay. The β-lactamase assay was carried out as previously described[13] with the following modifications. To assay blood from chicks 2 to 10 days old, the leg vein was pricked with a scalpel and 50 μl of blood was collected in a heparinized capillary tube (Fisher, Suwanee, GA). Then 25 μl of this sample was transferred to a 96-well plate containing 100 μl PBS. Various dilutions of purified β-lactamase (supplied by A. Bulychev and S. Mobashery, Wayne State University School of Medicine, Detroit, MI) were added to some wells prior to the addition of blood from non-transduced chicks to establish a standard curve. Plates were centrifuged for 10 min at 730 × *g*, and 25 μl of the supernatant was added to 75 μl of PBS. PADAC (Calbiochem, San Diego, CA; 100 μl of 20 μM solution in PBS) was added, and the change in absorbance at 560 nm was monitored at room temperature every 15 s for 30 min to 12 h with mixing between readings on a SPECTRAmax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

To assay β-lactamase in egg white, we transferred freshly laid eggs to a 4°C cooler and harvested them 1–2 months later. Egg white samples were mixed to include the thick and thin components in each sample. Aliquots of dilutions in PBS (10 μl) were transferred to a 96-well plate containing 100 μl of PBS supplemented with 1.5 μl of 1 M $NaH_2PO_4$ (pH 5.5) per well. After addition of 100 μl of 20 μM PADAC, values were measured as described for serum analysis.

Immunoblotting. Egg white (1.5–4 μl) was added to 30 μl of SDS-PAGE loading buffer, heated to 95°C for 5 min, separated on 12% SDS-PAGE and transferred to Immobilon P membranes (Millipore, Bedford, MA). β-lactamase was detected with 1:500 dilution of rabbit anti-β-lactamase (5 Prime→3 Prime, Boulder, CO) and 1:5,000 dilution of goat anti-rabbit IgG–horseradish peroxidase conjugate (Promega, Madison, WI). Immunoblots were visualized by the ECL System (Amersham, Piscataway, NJ) and membranes were exposed to film and quantified on a Molecular Devices Storm imaging system.

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